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Beneficial long-term antidiabetic actions of N- and C-terminally modified analogues of apelin-13 in diet-induced obese diabetic mice.

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Abbreviations

APJ, apelin receptor; AUC, integrated area under the curve; BMC, bone mineral content; BMD, bone mineral density; DIO, diet induced obese; DPP-4, dipeptidylpeptidase-4; DXA, Dual-energy X-ray absorptiometry; GIP, glucose-dependent insulintropic polypeptide; GLP-1, glucagon-like peptide-1; pGlu, pyroglutamyl; SGLT-2; sodium glucose co-transporter-2; T2DM, type 2 diabetes mellitus.

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Abstract

Aims: This study investigated the chronic effects of twice daily administration of stable apelin analogues, apelin-13 amide and (pGlu)apelin-13 amide, on metabolic parameters in glucose intolerant and insulin resistant diet-induced obese (DIO) mice fed a high-fat diet for 150 days. **Study Design & Methods:** Groups of mice received twice daily (09:00 and 17:00 h) injections of saline vehicle, apelin-13 amide, (pGlu)apelin-13 amide or exendin-4(1-39) for 28 days (all at 25 nmol/kg). Energy intake, body weight, non-fasting blood glucose, plasma insulin, glucose tolerance, metabolic response to feeding and insulin sensitivity together with pancreatic hormone content and biochemical parameters such as lipids and total GLP-1 were monitored. Dual-energy X-ray absorptiometry (DXA) analysis and indirect calorimetry were also performed. **Results:** Administration of apelin-13 amide, (pGlu)apelin-13 amide or exendin-4 significantly decreased bodyweight, food intake, blood glucose and increased plasma insulin compared with high-fat fed saline treated controls ($P<0.05$ and $P<0.001$). Additionally, all peptide treated groups exhibited improved glucose tolerance (oral and ip), metabolic responses to feeding and associated insulin secretion. (pGlu)apelin-13 amide also significantly improved HbA_{1c} and insulin sensitivity after 28 days. Both (pGlu)apelin-13 amide and exendin-4 increased bone mineral content and decreased respiratory exchange ratio (RER), whereas only (pGlu)apelin-13 amide increased energy expenditure. All treatment groups displayed reduced circulating triglycerides and increased GLP-1 concentrations, although only (pGlu)apelin-13 amide significantly reduced LDL-cholesterol, total body fat, and increased pancreatic insulin content. **Conclusion:** These data indicate the therapeutic potential of stable apelin-13 analogues with effects equivalent to or better than exendin-4.

1. INTRODUCTION

Metabolic syndrome presents an ever-increasing health challenge worldwide, thriving mainly because of surplus energy intake and lack of physical activity.¹ It is associated with visceral adiposity, impaired insulin sensitivity, hyperglycaemia, dyslipidaemia and hypertension.² Metabolic syndrome is associated with a 5-fold increase in risk for type-2 diabetes and 2-fold risk for cardiovascular disease.³ The prevalence of T2DM is rapidly increasing, especially in developing countries, in conjunction with adoption of a westernised lifestyle and increasing obesity rates.⁴ In addition to correction of lifestyle, diet modifications, increased exercise, abstinence from smoking and moderate consumption of alcohol,^{5,6} there is an unmet need for novel pharmaceutical agents to better help counter diabetes progression and complications.

Glycaemic management in type 2 diabetes requires a complex strategy and patient stratification, as a widening array of pharmacological agents are now available.^{7,8} These include, metformin, sulphonylureas, thiazolidinediones, DPP-4 inhibitors, GLP-1 mimetics, SGLT-2 inhibitors and, if all else fails to achieve acceptable blood glucose control, therapy with insulin.⁹ Approximately 80% of individuals with T2DM are overweight or obese,¹⁰ and intensive lifestyle intervention could improve fitness, glycaemic control and cardiovascular risk factors for relatively small changes in body weight.¹¹ However due to ineffective energy balance, type 2 diabetic subjects who are obese are more likely to require combination drug therapy.¹² Thiazolidinediones (TZDs) are more effective in patients with a high BMI, but further weight gain, as with insulin, makes them a much less attractive option.¹³ GLP-1 receptor agonists offer a distinct advantage of weight reduction, but nausea is a drawback and occasional cases of acute pancreatitis have been reported.¹⁴⁻¹⁵

In order to bring forward new and more effective approaches to diabetes therapy, we have explored the potential of apelin-13 analogues to lower blood glucose and decrease body weight.¹⁶ Apelin is an adipokine, secreted from adipocytes which act on APJ receptors that are widely distributed in various tissues including the heart, lung, liver, brain, adipose tissue, gastrointestinal tract and bone.¹⁷ Apelin conveys multiple biological actions, including regulation of feeding behaviour, glucose utilisation, insulin secretion and blood pressure.¹⁸

To harness the potential of apelin and APJ receptors as target for diabetes, we have developed a series of stable analogues of apelin-13, which stimulate insulin secretion and cellular glucose uptake *in vitro*.¹⁶ In acute *in vivo* studies in mice, these analogues also improved glucose tolerance and inhibited feeding, exhibiting protracted effects.¹⁶ In the present study, we have examined the effects of chronic twice daily administration of two of the most promising analogues, apelin-13 amide and (pGlu)apelin-13 amide, in comparison to the established incretin mimetic exendin-4(1-39), on metabolic control in a high-fat fed diet induced obese (DIO) mouse model of obesity-diabetes.

2. METHODS

2.1 Animals

Male NIH Swiss mice (Harlan Ltd, Blackthorne, UK) were housed individually in an air-conditioned room ($22 \pm 2^{\circ}\text{C}$) with relative humidity of $51 \pm 5\%$ and a 12 h light: dark cycle (08:00 – 20:00 h). Animals were maintained on high-fat diet (45% fat, 20% protein, 35% carbohydrate; percent of total energy 26.15 kJ/g; Dietex International Ltd., Witham, UK) for 150 days to produce a model of diet induced obesity (DIO) diabetes. Normal lean mice

received a standard rodent diet (10% fat, 30% protein, 60% carbohydrate; percent of total energy 12.99 kJ/g, Trouw Nutrition, Cheshire, UK). DIO mice exhibited increased body weight and elevated non-fasting blood glucose compared with mice receiving a standard rodent chow. Drinking water was provided *ad libitum* and all animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63EU for animal experiments and approved by University of Ulster Animal Welfare and Ethical Review Committee. No adverse reactions were observed during the treatment period.

2.2 Peptide administration

Apelin-13 amide, (pGlu)apelin-13 amide and exendin-4(1-39) were purchased from (EZBiolabs, Carmel, IN, USA) with a purity of >95%. Purity and structures were confirmed using RP-HPLC and in-house matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS), as previously described.¹⁹ To improve the stability, native apelin-13 was either amidated at the C-terminus or a pyroglutamate was added at the N-terminus as previously described.²⁰ Animals received twice daily intraperitoneal (i.p.) injections (0900 and 1700 h) of either 0.9% saline vehicle (lean and high fat-fed control group) or of apelin-13 amide, (pGlu)apelin-13 amide or exendin-4(1-39) (each at 25 nmol/kg bw) over a 28-day treatment period. This dose was chosen on the basis of previous experience with peptides.^{16,21}

2.3 Measurement of metabolic effects

Food intake, body weight, non-fasting blood glucose and plasma insulin were measured at intervals of 2-3 days throughout the run-in and the 28-day treatment periods. Blood samples

were collected from the cut tail vein of conscious mice. Blood glucose was measured using an Acensia Contour meter (Bayer Healthcare, Newbury, UK). For insulin, blood was collected in heparin/fluoride microvette (Sarstedt, Numbrecht, Germany), centrifuged (13000 x g, 3 min) and plasma stored at -20°C prior to measurement by modified dextran coated charcoal radioimmunoassay.²²

2.4 Glucose tolerance and insulin sensitivity

Following the 28-day treatment period, glucose tolerance (18 mmol/kg bw; i.p. and oral) and metabolic response to feeding, were examined in overnight (12 h) fasted lean and DIO mice treated with either peptides or saline as described previously.²³ Whole body insulin sensitivity tests (25 U/kg bw) were carried as described previously using fed mice.²¹

2.5 Energy intake and body composition

Comprehensive Laboratory Animal Monitoring System (CLAMS) metabolic chambers (Columbus Instrument, Columbus, OH, USA) were used to measure indirect calorimetry and energy expenditure in the peptide treated and lean control groups after 28 days of treatment as described previously.^{21,24} Respiratory Exchange Ratio (RER) was calculated by dividing VCO_2 by VO_2 . Energy expenditure (EE) was calculated using the equation $\text{EE} = 3.815 + 1.232 \times \text{RER} \times \text{VO}_2$. Body composition, bone mineral content and total fat mass were measured by DXA scanning (Piximus Densitometer, Fitchburg, WI, USA).

2.6 Pharmacokinetic study with radiolabelled pGlu(Tyr¹³)apelin-13 amide

The pGlu(Tyr)apelin-13 amide analogue was labeled with ^{125}I according to iodogen method, as described previously.²⁵ Briefly, 35 μl of H_2O , 5 μl of 2 M AcOH, 10 μl 1 mM peptide (pGlu(Tyr¹³)apelin-13) and 5 μl of Na- ^{125}I (100 mCi/ml stock) was added to iodogen coated tubes (5 μg), and vortexed. The reaction mixture was incubated on ice for 30 min with gentle agitation once every min. The reaction was terminated by transferring to a new polypropylene Eppendorf tube (1.5 ml) washed with 500 μl of sodium phosphate buffer (50 mM) and purified using reverse-phase HPLC. The eluent was collected in 1 ml fractions, radioactive counts (CPM) analyzed using a 1470 Multigamma counter (Perkin Elmer Wallac Wizard 1470, San Diego, CA, USA) and highest radioactivity peak was selected for *in vivo* experiments. The ^{125}I -labelled apelin analogue was administered to mice by i.p. injection at 4.1 nmol/kg (5 μCi) and blood was collected at various time intervals (from 0-24 h) into chilled fluoride/heparin microcentrifuge tubes (Starstedt, Numbrecht, Germany) and centrifuged immediately for 2 min at 12,000 g at 4°C. The resulting plasma was aliquoted in fresh low-protein binding Eppendorf tubes and stored at -20°C. Correction for free iodine, a product of intracellular dehalogenation, was performed by determining radioactivity of pellet after precipitation with 10% final concentration of trichloroacetic acid (TCA). The half-life of the peptide was calculated from the equation $\ln(2)/\lambda Z$.

2.7 Biochemical analyses

Glycated haemoglobin (HbA_{1c}) was measured using point of care A1CNow⁺ kit (PTS Diagnostic, IN, USA). Lipid profile including analysis of total cholesterol, triglycerides, high density lipoprotein (HDL-C) and low-density lipoprotein (LDL-C) concentrations by I-Lab 650 clinical analyser (Instrumentation Laboratory, Warrington, UK) as described previously.²⁶ Serum amylase (Amylase assay kit, Abcam, UK) and total GLP-1 (ELISA, Millipore, UK) were measured from terminal plasma as per manufacturer's protocols.

Pancreatic tissue was excised at the end of the study, weighed and processed for measurement of insulin content following extraction with ice-cold acid ethanol.^{21,27}

2.8 Statistical analysis

All results were analysed using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA) and expressed as mean \pm S.E.M. Data were compared using the Student's t-test, one-way and two-way Analysis of Variance (ANOVA) followed by Student–Newman–Keuls *post-hoc* test wherever applicable. Area under the curve (AUC) was calculated using the trapezoidal rule with baseline correction. Groups of data were considered to be statistically significant if $p < 0.05$.

3. RESULTS

3.1 Effects of apelin-13 amide and (pGlu)apelin-13 amide on metabolic status in DIO mice

Twice daily administration of apelin-13 related peptide analogues resulted in decreased bodyweight ($p < 0.05$) which was more marked than observed with exendin-4(1-39) (Fig. 1A). A greater % bodyweight change was noted with apelin analogue treatment ($p < 0.05$ to $p < 0.001$; Fig. 1B). Mice treated with (pGlu)apelin-13 amide displayed significantly decreased cumulative energy intake ($p < 0.05$; Fig. 1C) and a sustained decrease in non-fasted blood glucose ($p < 0.05$ to $p < 0.01$; Fig. 1D) which was mirrored by increased non-fasting insulin ($p < 0.05$ and $p < 0.01$; Fig. 1E) by day 9. Apelin-13 amide and exendin-4 had no significant effects on energy intake, or non-fasting plasma insulin concentrations (Fig. 1C,E) but exendin-4 had an antihyperglycaemic effect similar to (pGlu)apelin-13 amide (Fig. 1D).

3.2 Effects of apelin-13 amide and (pGlu)apelin-13 amide on glucose tolerance and insulin sensitivity in DIO mice

Treatment with apelin-13 amide, (pGlu)apelin-13 amide or exendin-4(1-39) for 28 days significantly decreased blood glucose excursion at 15, 30, 60 and 105 min post i.p. or oral glucose tolerance test ($p < 0.05$ to $p < 0.001$; Fig. 2A and Fig. 2E). This was accompanied by potentiated glucose-induced insulin secretion ($p < 0.05$ to $p < 0.01$; Fig. 2C and Fig. 2G). Integrated responses (area under curve, AUC) to i.p. and oral glucose challenge, showed parallel increase in plasma insulin ($p < 0.01$ to $p < 0.001$; Fig. 2D and Fig. 2H) and was associated with equivalent blood glucose reductions ($p < 0.01$ to $p < 0.001$; Fig. 2B and Fig. 2F) compared to DIO saline treated mice.

In overnight fasted mice, chronic administration of (pGlu)apelin-13 amide or exendin-4 for 28 days resulted in significant reductions in blood glucose excursion ($p < 0.05$; Fig. 3A) following 15 min feeding compared to high-fat-fed saline treated mice. However, only apelin-13 amide and (pGlu)apelin-13 amide exerted a significant stimulatory effect on plasma insulin concentrations ($p < 0.05$; Fig. 3B). Additionally, glucose-lowering effects of exogenous insulin were enhanced in all groups but were statistically significant in only (pGlu)apelin-13 amide ($p < 0.05$; Fig. 3C) treated mice.

3.3 Effects of apelin-13 amide and (pGlu)apelin-13 amide on glycated haemoglobin and lipid biochemistry, GLP-1, amylase and pancreatic insulin content

Saline-treated DIO mice showed significantly higher levels of glycated haemoglobin compared to lean mice ($p < 0.01$; Fig 4A). (pGlu)apelin-13 amide administration significantly reduced glycated haemoglobin levels compared with saline-treated DIO control mice ($p < 0.05$; Fig. 4A). Elevated concentrations of circulating plasma total cholesterol, exhibited

by DIO mice ($p < 0.01$; Fig. 4B) was normalised by administration of exendin-4 ($p < 0.05$), but not significantly by apelin analogues (Fig. 4B). All peptide treatment significantly reduced circulating triglycerides ($p < 0.05$ to $p < 0.01$; Fig. 4C). No treatment-related changes in HDL cholesterol response were observed in DIO mice (Fig. 4D). However, treatment with (pGlu)apelin-13 amide significantly reduced LDL cholesterol ($p < 0.01$; Fig. 4E) compared to DIO mice controls. In contrast, exendin-4(1-39) significantly increased LDL-cholesterol compared to the normal lean mice ($p < 0.01$; Fig. 4E).

Circulating amylase concentrations were comparable in all groups, demonstrating no adverse effect of treatment on the integrity of exocrine pancreas (Fig. 5A). Terminal plasma samples from all peptide treated groups showed significantly increased ($p < 0.01$) circulating total GLP-1 concentrations (Fig. 5B), compared to saline-treated DIO controls. Mice treated with (pGlu)apelin-13 amide had a significantly higher pancreatic insulin content ($p < 0.01$; Fig. 5C) compared to healthy lean control mice. However, no significant differences in pancreatic insulin content were observed between treatment groups and saline-treated DIO mice.

3.4 Effects of apelin-13 amide and (pGlu)apelin-13 amide on bone composition and fat mass

Mice treated with (pGlu)apelin-13 amide showed a reduced final body weight (Fig. 5D) and percentage body fat mass ($p < 0.05$; Fig. 5E) compared to saline-treated DIO mice. Exendin-4(1-39) and (pGlu)apelin-13 amide both significantly increased bone mineral content (BMC) compared with high-fat saline treated controls, as well as lean controls ($p < 0.05$; Fig. 5F). No significant effect on bone mineral density (BMD) was observed (data not shown).

3.5 Assessing effects of apelin-13 amide and (pGlu)apelin-13 amide on energy expenditure by indirect calorimetry

No significant changes in oxygen consumption (Suppl. Fig. 1A-B) or carbon dioxide production (Suppl. Fig. 1C-D) were observed in peptide treated versus saline-treated DIO groups. In contrast, both (pGlu)apelin-13 amide and exendin-4(1-39) treated mice showed significantly increased average RER compared to DIO saline-treated controls ($p < 0.05$ and $p < 0.01$; Suppl. Fig. 2AB). This effect was demonstrated in both the light and dark cycles (Suppl. Fig. 2C-D). (pGlu)apelin-13 amide treatment was also associated with an increase in overall average energy expenditure compared to saline-treated DIO mice ($p < 0.05$; Suppl. Fig. 3B) evident mainly in the dark cycle ($p < 0.05$; Suppl. Fig. 3D). In addition, (pGlu)apelin-13 amide treatment caused mice to rear more often (Z axis beams broken) ($p < 0.05$; Suppl. Fig. 4F). No differences in any other ambulatory behaviour were observed (Suppl. Fig. 4A-E). The mice administered (pGlu)apelin-13 amide, had a significantly reduced accumulated food and energy intake over 24 h compared to saline-treated DIO mice ($p < 0.05$; Suppl. Fig. 5A and 5B). There were no significant effects observed in the number of feeding bouts of peptide-treated groups compared to saline-treated DIO mice ($p > 0.05$; Suppl. Fig. 5C).

3.6 Pharmacokinetic profile using radiolabelled pGlu(Tyr)apelin-13 amide

Following an i.p. injection of radiolabelled pGlu(Tyr-I¹²⁵)apelin-13 amide an initial radioactivity peak was reached in mouse plasma after 60 min (8.5 pM) but this rose to a new peak after 180 min (12.1 pM). The amount of radioactivity declined sharply thereafter reaching a level close to basal by 480 min (0.9 pM) and was completely gone by 24 h (1440 min), indicating a half-life of approximately 1 h.

4. DISCUSSION

Defective insulin secretion and impaired insulin action are major factors contributing to metabolic disarray and raised blood glucose concentrations in type 2 diabetes.^{8,9} Lack of suppression of glucagon together with dysfunctional participation of the incretin hormones GLP-1 and GIP further compounds the problem.^{28,29} The challenge for successful treatment of diabetes is to address these and other basic mechanisms that are defective in the disease state.

Recent studies in our laboratory suggest that the adipokine apelin may have such therapeutic potential despite being rapidly cleaved and rendered inactive by ACE enzymes.¹⁶ We have found that modification of native apelin-13 by the addition of an N-terminal pyroglutamate motif and/or an amide group at C-terminus conferred enzyme resistance and prolonged plasma half-lives.¹⁶ Compared with the parent apelin-13 peptide, such modifications also significantly augmented effects on insulin release, adipocyte glucose uptake, and *in vivo* acutely induced satiety and evoked protracted glucose lowering in both lean and DIO mice.

In the present study, long-term metabolic effects of two of these stable analogues, apelin-13 amide and (pGlu)apelin-13 amide, were compared to the established antidiabetic agent exendin-4(1-39) using DIO mice that exhibited obesity, insulin resistance, and diabetes. Twice daily administration of (pGlu)apelin-13 amide or exendin-4 produced distinct reductions in non-fasting glucose, accompanied by hyperinsulinaemia, elevated circulating GLP-1, increased pancreatic insulin stores and enhanced glucose-induced insulin secretion. Such effects are well established for GLP-1 mimetics^{30,31} and accord also with our previous data showing positive actions of apelin-13 on insulin secretion, cellular glucose uptake and as modulator of incretins.¹⁶ Administration of apelin-13 amide also induced a range of beneficial actions in DIO mice but the effects were generally inferior to those observed with additional N-terminal pyroglutamate modification, which seemed to confer greater stability and bioactivity *in vitro*.

Consistent with marked therapeutic potential, DIO mice treated with both apelin-13 analogues showed significant enhancement of intraperitoneal and oral glucose tolerance, which was associated with elevated plasma insulin concentrations. Such effects are likely to reflect direct beneficial actions of the peptides on pancreatic beta cells combined with possible effects also mediated via increased cellular glucose uptake and activity of the enteroinsular axis. In support of this view, treatment with of (pGlu)apelin-13 amide, apelin-13 amide or exendin-4 increased the insulin response and lowered the glycaemic excursion following feeding. Benefits in terms of insulin sensitivity, possibly mediated by actions of GLP-1 on glucose uptake in adipocytes and skeletal muscle,^{32,33} might also contribute to glucose lowering, but insulin action, as measured by glucose lowering following acute insulin injection, was only enhanced in DIO mice treated with (pGlu)apelin-13 amide. HbA_{1C} levels were only improved significantly at the end of the study in this same group, indicative of superior antidiabetic activity compared with both apelin-13 amide or exendin-4.

As expected, prolonged high-fat consumption induced weight gain, deposition of adipose reserves and elevated circulating triglycerides.^{21,34} Treatment with either apelin-13 analogue or exendin-4, significantly reduced body weight and circulating triglyceride concentrations with (pGlu)apelin-13 amide having an additional positive effect of reducing LDL-C and restoring body fat content to proportions similar to healthy lean mice. This reduced adiposity could contribute to improved insulin sensitivity as shown previously but no changes in HDL cholesterol were observed. Others have demonstrated that GLP-1 and exendin-4(1-39) significantly decreased total cholesterol,³⁵ which is consistent with our results. It is also notable in the present study that concentrations of circulating amylase were similar to lean controls, suggesting no adverse reaction indicative of pancreatitis as claimed previously with some GLP-1 analogues.³⁶

Interestingly, the decreased body weights observed in all treatment groups were only accompanied by measurable inhibition of food intake in (pGlu)apelin-13 amide treated mice. Such action itself could in part be due to high co-localisation of APJ receptor with proopiomelanocortin (POMC) in the hypothalamic arcuate nucleus (Arc) which secretes α -melanocyte-stimulating hormone (α -MSH), a strong suppressor of appetite.³⁷ Exendin-4 is noted to induce weight loss in humans, albeit its effects are less obvious and reproducible in animal models as observed by ourselves and others.^{38,39} Since accurate measurement of food intake is difficult to record in normally housed freely fed mice, we would not rule out small contribution of decreased energy intake to the weight loss in all groups of mice. Nevertheless, it would seem likely that increase energy expenditure makes an important contribution to the declining weight, especially in the (pGlu)apelin-13 amide treated group.

Both O₂ consumption and CO₂ production were increased in DIO mice, indicating enhanced whole body metabolism.⁴⁰ In support of key metabolic action, the decrease of fat mass observed in mice treated with (pGlu)apelin-13 amide was associated with increases in energy expenditure and spontaneous locomotor activity. Interestingly, the average RER was higher in both the dark and light phase in (pGlu)apelin-13 amide treated mice. This is consistent with a switch from fat metabolism (RER=0.75) towards protein (RER=0.9) and/or carbohydrate (RER=1.0) metabolism, reflecting the depleted fat depots in treated mice. Increased body weight and fat mass impacts on bone deterioration.⁴¹ It is notable that GLP-1 has numerous effects apart from antidiabetic actions, including osteogenic properties.³⁹ Administration of (pGlu)apelin-13 amide and exendin-4(1-39) exerted significant positive effects on bone mineral content (BMC). The present findings in mice were consistent with those of rats fed a high-fat diet, as administration of GLP-1 and exendin-4(1-39) caused significant increases in BMC levels,⁴² indicating extra-pancreatic effects of (pGlu)apelin-13 amide.

In conclusion, these observations indicate that chronic treatment of DIO mice with apelin-13 amide and in particular (pGlu)apelin-13 amide for 28 days exerted multiple beneficial effects similar to or better than treatment with the well-established GLP-1 therapeutic, exendin-4(1-39). Other research indicates that apelin exerts important anti-inflammatory and cardioprotective effects, which could be helpful also in countering diabetes complications. These attributes make apelin-13 an attractive drug candidate and further studies are warranted to explore the antidiabetic effects of stable analogues and their potential for use in the treatment of the disease.

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Figure Legends

Figure 1. Chronic effect of twice daily administration of exendin-4(1-39), apelin-13 amide or (pGlu)apelin-13 amide (each at 25 nmol/kg bw) on (A) body weight, (B) % body weight change, (C) cumulative energy intake, (D) non fasting blood glucose and (E) plasma insulin during 28-day treatment. The black horizontal bar represents the treatment period. Values represent mean \pm S.E.M. (n=8) where *p<0.05, **p<0.01 and ***P<0.001 is compared to high-fat fed saline treated mice, $\nabla\nabla$ p<0.01 and $\nabla\nabla\nabla$ p<0.001 is compared to lean mice fed on a normal diet.

Figure 1

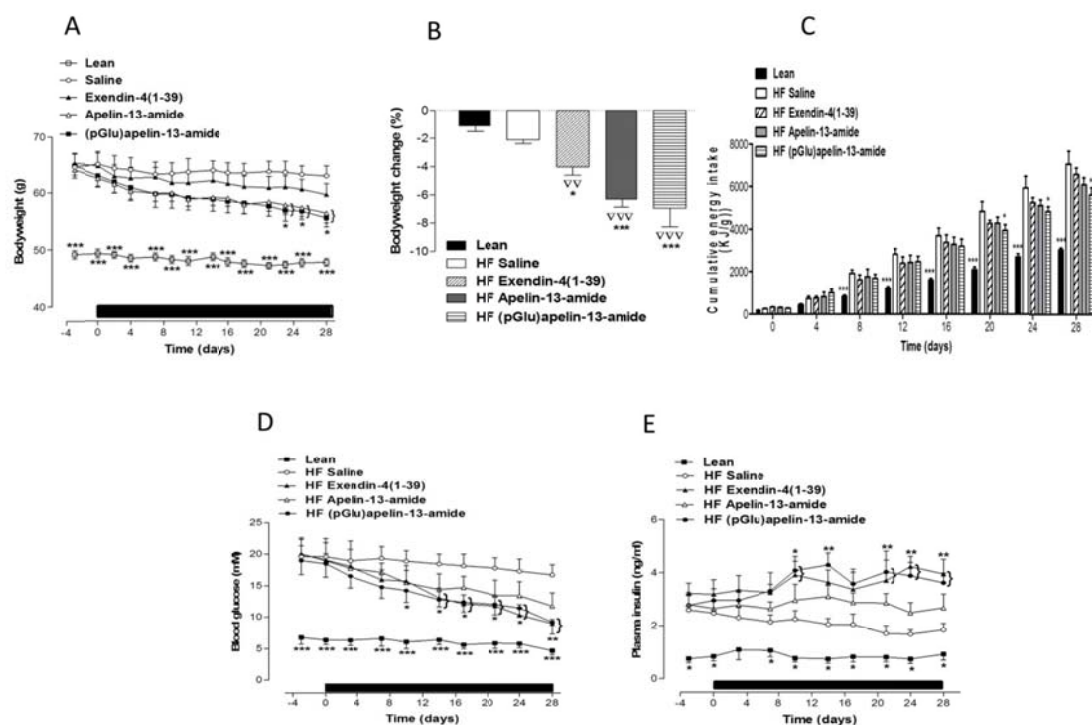


Figure 2. Effect of twice daily administration of exendin-4(1-39), apelin-13 amide or (pGlu)apelin-13 amide on blood glucose and plasma insulin in response to (A-D) an intraperitoneal or (E-H) oral glucose challenge in high fat diet fed mice. Tests were performed after 28 days of twice-daily i.p. administration of saline, exendin-4 or apelin analogues (each at 25 nmol/kg body weight). Mice were fasted for 18 h previously. Blood glucose (A and E) and plasma insulin concentrations (C and G) were measured before and after i.p. (A, C) or oral (E, G) administration of glucose (18 mmol/kg body weight). Blood glucose and integrated plasma insulin response (area under the curve; AUC, 0-105 min) are also included. Values represent mean \pm S.E.M. (n=8) where *p<0.05, **p<0.01 and ***p<0.001 is compared to high fat fed saline treated mice, ∇ p<0.05, $\nabla\nabla$ p<0.01 and $\nabla\nabla\nabla$ p<0.001 is compared to lean mice fed on a normal diet.

Figure 2

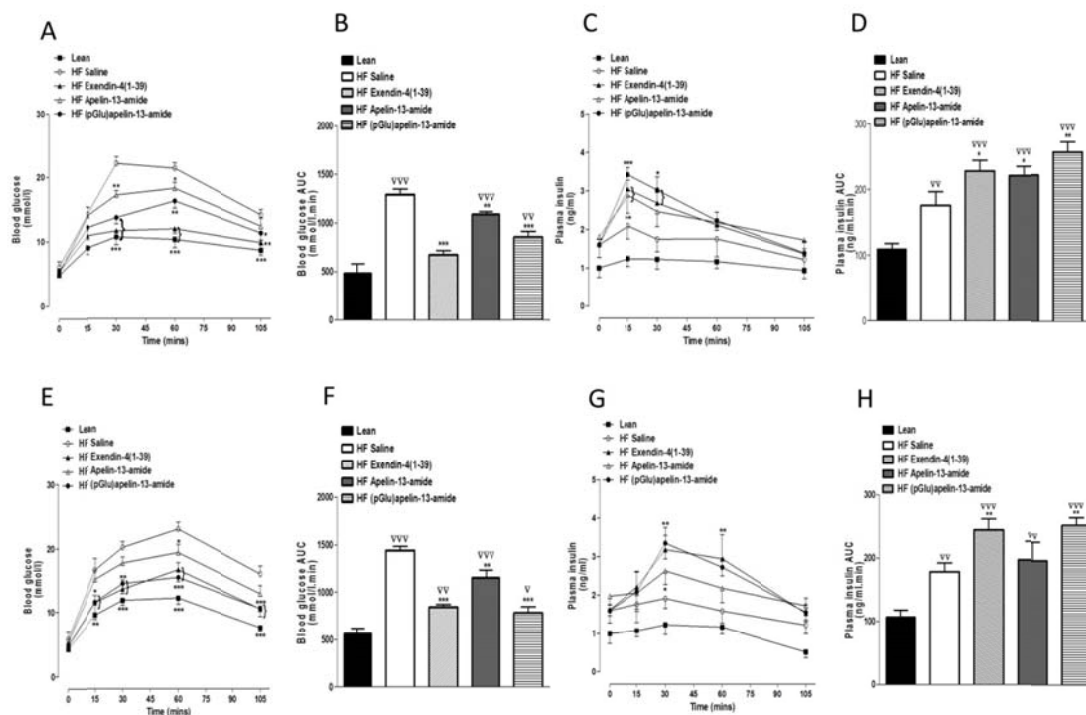


Figure 3. Effect of twice daily administration of exendin-4 (1-39), apelin-13 amide or (pGlu)apelin-13 amide on (A) blood glucose and (B) plasma insulin (B) in response to 15 min feeding and (C) insulin sensitivity in high fat diet fed mice. Tests were performed after 28 days of twice-daily i.p. administration of saline, exendin-4 or apelin analogues (each at 25 nmol/kg body weight). Mice were fasted for 18 h previously and given free access to a normal chow diet for 15 min. Blood glucose and plasma insulin concentrations were measured at t=0, 15, 30, 60 and 105 min and time of feeding is represented by the black horizontal bar. Blood glucose and plasma insulin Area under the curve (AUC) values are also included. For insulin sensitivity, insulin (25 U/kg body weight) was administrated by i.p. injection to fed mice at t=0 min with blood glucose measured at t=30 and 60 min. The % blood glucose and Area Above the Curve (AAC) values (C) for 0-60 min post-injection are

shown. Values represent the mean \pm S.E.M. (n=8) where * p <0.05, ** p <0.01 and *** p <0.001 is compared to high fat fed saline treated mice, ∇p <0.05, $\nabla\nabla p$ <0.01 and $\nabla\nabla\nabla p$ <0.001 is compared to normal lean mice.

Figure 3

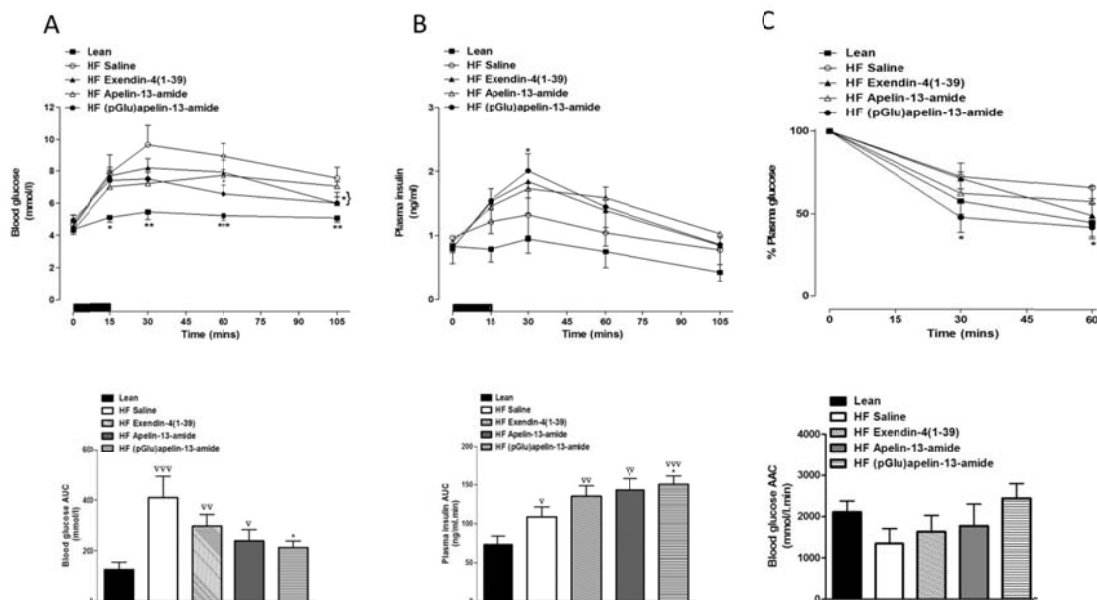


Figure 4. Effects of twice daily administration of saline, exendin-4(1-39), apelin-13-amide or (pGlu)apelin-13-amide (each at 25 nmol/kg bw) on (A) HbA_{1c}, recorded by Bayer A1CNow⁺ multi-test system (B) plasma total cholesterol, (C) plasma triglycerides, (D) HDL cholesterol and (E) LDL cholesterol after 40 days of treatment of high fat fed and lean control mice. Values represent the mean \pm S.E.M. (n=8) where * p <0.05, ** p <0.01 and *** p <0.001 is compared to high-fat fed saline treated mice, ∇p <0.05 and $\nabla\nabla p$ <0.01 is compared to normal lean mice.

Figure 4

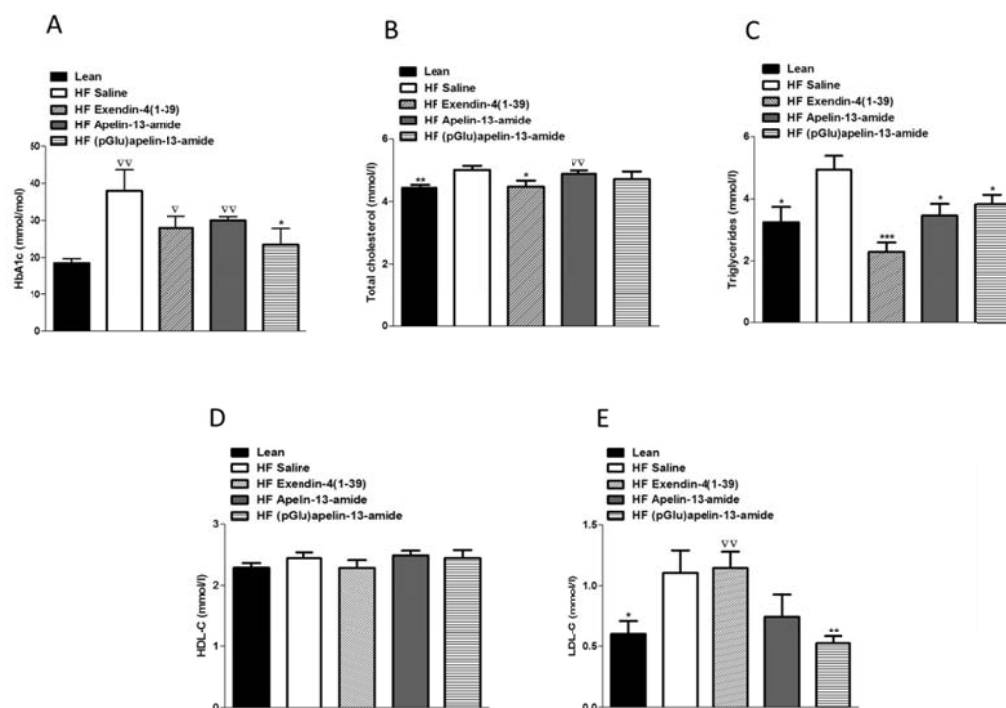
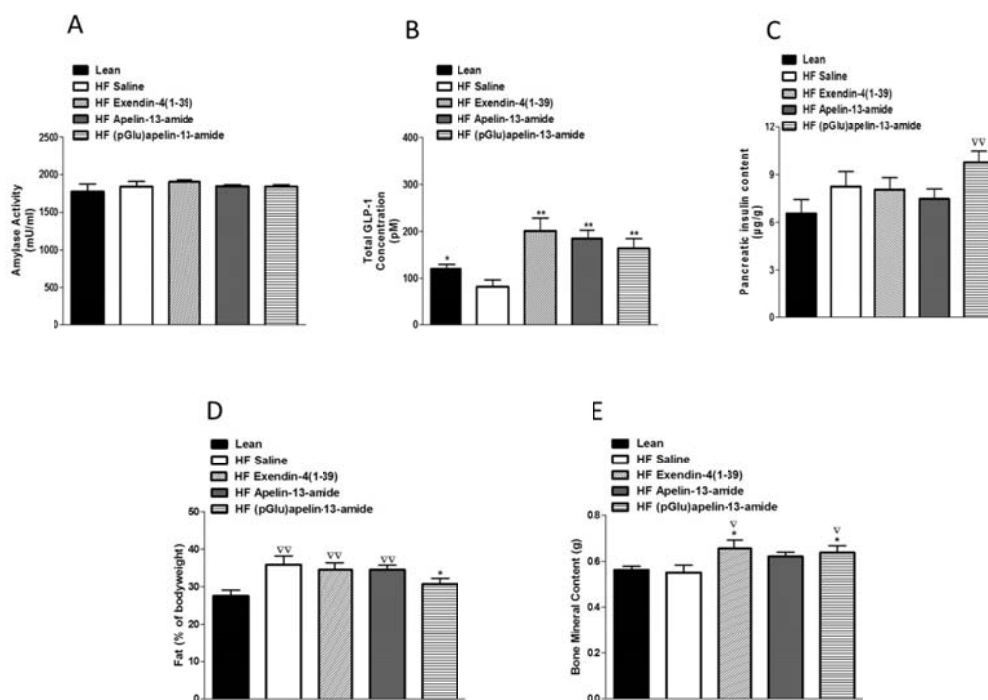


Figure 5. Effects of twice daily i.p. administration of saline, exendin-4(1-39), apelin-13amide or (pGlu)apelin-13amide (each at 25 nmol/kg bw) on (A) α -amylase activity, (B) plasma GLP-1, (C) pancreatic insulin content, and (D) body weight (at end of study) (E) and fat mass (%), and (F) bone mineral content measured by DXA scanner. Observations were made after 40 days of treatment of high fat fed and lean control mice. Values represent the mean \pm S.E.M. (n=8) where * p <0.05, ** p <0.01 and *** p <0.001 is compared to high-fat fed saline treated mice, ∇ p <0.05, $\nabla\nabla$ p <0.01 and $\nabla\nabla\nabla$ p <0.001 is compared to normal lean mice.

Figure 5



Supplementary Figure Legends

Supplementary Figure 1. Effects of twice daily i.p. administration of saline ((0.9% w/v) NaCl), exendin-4(1-39), apelin-13 amide or (pGlu)apelin-13 amide (each at 25 nmol/kg bw) on (A, B) O_2 consumption, and (C, D) CO_2 production. Mice were placed in CLAMS metabolic chambers for 24 h (12 h dark period as shown by the black bar), O_2 consumption and CO_2 production were measured for 30 sec at 25 min intervals. Values represent the mean \pm S.E.M. (n=6) where **p<0.01 is compared to high fat fed saline treated mice.

Supplementary Figure 2. Effects of twice-daily i.p. administration of saline, exendin-4(1-39), apelin-13 amide or (pGlu)apelin-13 amide (each at 25 nmol/kg bw) on (A) respiratory exchange ratio (RER). Following 35 days treatment mice were placed in CLAMS metabolic chambers for 24 h (12 h dark period as shown by the black bar). RER was calculated by

dividing VCO_2 by VO_2 . (B) Average RER (C), RER in the light, and (D) dark cycles are also included. Values represent the mean \pm S.E.M. (n=6) where * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ is compared to high fat fed saline treated mice.

Supplementary Figure 3. Effects of twice-daily i.p. administration of saline, exendin-4(1-39), apelin-13 amide or (pGlu)apelin-13 amide (each at 25 nmol/kg bw) on (A) energy expenditure. Mice were placed in CLAMS metabolic chambers for 24 h (12 h dark period as shown by the black bar) and energy expenditure calculated using RER with the following equation: $(3.815 + 1.232 \times RER) \times VO_2$. (B) Average energy expenditure (C), energy expenditure in the light, and (D) and dark cycles are also included. Values represent the mean \pm S.E.M. (n=6) where * $p<0.05$ is compared to high fat fed saline treated mice.

Supplementary Figure 4. Effects of twice daily i.p. administration of saline, exendin-4(1-39), apelin-13-amide or (pGlu)apelin-13 amide (each at 25 nmol/kg bw) following 35-days administration on locomotor activity using optical beams. Mice were placed in CLAMS metabolic chambers for 24 h (12 h dark period as shown by the black bar). Activity counts in X-axis (lateral) (A-D) and Z-axis (vertical) (E-F) were recorded every min for the duration. Values represent the mean \pm S.E.M. (n=6) where * $p<0.05$ is compared to high fat fed saline treated mice.

Supplementary Figure 5. Effects of twice daily i.p. administration of saline, exendin-4(1-39), apelin-13-amide or (pGlu)apelin-13 amide (each at 25 nmol/kg bw) following 35 days administration on food intake. Mice were placed in CLAMS metabolic chambers for 24 h and (A) food intake (B), energy intake, and (C) feeding bouts were measured for the duration. Values represent the mean \pm S.E.M. (n=6) where * $p<0.05$ and ** $p<0.01$ compared to high-fat

fed saline treated mice and $\nabla p < 0.05$, $\nabla\nabla p < 0.01$ and $\nabla\nabla\nabla p < 0.001$ is compared to lean mice fed on a normal diet